EFFECT OF BRASSINOSTEROID/S ON THE METAL INDUCED CHANGES IN SOME LEGUMES

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SYED AIMAN HASAN

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Dedicated

To

My Parents

(Mr. Mahfoozul Hasan and Mrs. Rana Mahfooz)

Prof. AQIL AHMAD
M.Phil., Ph.D.
PD.Res.Train (Denmark)

Plant Physiology Section
Department of Botany
Aligarh Muslim University
Aligarh-202002, U.P., India
E-mail: aqil_ahmad@lycos.com

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Certificate

This is to certify that the dissertation entitled, ***"Effect of Brassinosteroid/s on the metal induced changes in some legumes"*** *submitted in partial fulfillment of the requirements for the degree of Master of Philosophy in Botany is a bonafied research work carried out under my supervision at the Aligarh Muslim University, Aligarh, India by* ***Miss Syed Aiman Hasan*** *and that no part of it has been submitted for any other degree or diploma.*

(Prof. Aqil Ahmad)
Research Supervisor

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Aiman

(S. Aiman Hasan)

CONTENTS

Introduction

CHAPTER 1

INTRODUCTION

Cadmium is one of the most toxic heavy metals in contaminated crop environment (Wagner, 1993). Its concentration in non-polluted soil solution ranges from 0.04 to 0.32 mM. However, the soil solution with 0.32 to about 1 mM Cd may be categorized as polluted to moderate level (Sanita-di Toppi, 1990).

Cadmium is not an essential nutrient but at higher concentrations it inhibits plant growth. The metal is recognized as an extremely toxic pollutant due to its higher solubility in water (Pinto *et al.*, 2004). It retards the biosynthesis of chlorophyll (Singh and Tewari, 2003), alters water balance (Barcelo and Poschenrider, 1990), decreases the activities of various enzymes (Siedlecka *et al.*, 1997; Goucia *et al.*, 2003), favours stomatal closure (Poschenrieder *et al.*, 1989) and checks photosynthesis (Sheoran *et al.*, 1990; Chaug and Sawhrey, 1999). Cadmium stress reduces the uptake of essential mineral nutrients and also affects the activity of ATPase that decreases normal H^+/K^+ exchange at the level of plasma membrane (Obata *et al.*, 1996).

Brassinosteroids (BRs), a new class of phytohormones that occurs in all plant parts, including root (Bajguz and Treytn, 2003) elicits a wide range of physiological responses in plants, including stem elongation, pollen tube growth, leaf bending and epinasty, root growth inhibition, induction of ethylene biosynthesis, activation of proton pump, xylem-differentiation, synthesis of nucleic acid and of proteins and also speeds up the rate of

photosynthesis (Clouse and Sasse, 1998; Li and Chory, 1999; Khripach *et al.*, 2003; Hayat and Ahmad, 2003; Sasse, 2003; Yu *et al.*, 2004). It is proposed that the changes induced by BRs are mediated through the repression and/or depression of specific genes (Felner, 2003). Besides this, BRs are also recognized to have an ameliorative role in plants, subjected to various biotic and abiotic stresses (Clouse and Sasse, 1998). The treatment of the plants of rice and tomato (Kamuro and Takatsuto, 1991), maize (He *et al.*, 1991), cucumber (Katsumi, 1991) and brome grass (Wilen *et al.*, 1995) with BRs improved their capacity of resistance to low temperature. Similarly, BRs increased the degree of tolerance to high temperature, in wheat (Kulaeva *et al.*, 1991) and brome grass (Wilen *et al.*, 1995). Brassinosteroids also countermend the drought stress in sugarbeet (Schilling *et al.*, 1991), moisture stress in wheat (Sairam, 1994) and also favoured seed germination and seedling growth in *Eucalyptus* (Sasse *et al.*, 1995) and rice (Anuradha and Rao, 2001) under saline conditions BRs activated antioxidative enzymatic defence system in rice seedlings, grown under salt stress (Nunez *et al.*, 2003).

The present study was carried out with an aim to explore and elaborate the ameliorative role of brassinosteroids against heavy metal stress, more specifically the cadmium stress and to work out some remedial measures to overcome the toxicity of cadmium in plants.

Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

Literature provides extensive information about the toxicity of heavy metals on the growth and development of higher plants. Although, any of the known heavy metals may be toxic to plants at an elevated level, but Cd^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+} and Pd^{2+} cause phytotoxicity in the soil (Foy *et al.*, 1978). The concentration of these metals in uncontaminated soils range from 5 to 50 ppm (Schactchabel *et al.*, 1984) and in the plants, grown in such soils, the range is between 0.4-3 ppm (El-Bassam, 1978). Out of the major heavy metals Cd is an ever increasing industrial pollutant, particularly in areas associated with smelting of Zn and heavy road traffic (Ernst, 1980). In most of the environmental conditions Cd enters first into the roots, consequently they are the sight that first experience the Cd damage. In root tip cells of *Allium cepa*, Cd damaged nucleoli (Liu *et al.*, 1995).

Cadmium flow to human beings is largely through cereals, vegetables, fruits (Wagner, 1993) and edible parts of other plants. Excess of Cd causes a number of toxic symptoms in plants e.g. growth retardation, inhibition of photosynthesis, chlorosis (Das *et al.*, 1997), reduced rhizosphere (Vesper *et al.*, 1978, Vigue *et al.*, 1981), induction and inhibition of enzyme, altered stomatal action (Barcelo *et al.*, 1986, Poschenrieder, 1990) water relations, efflux of cations and generation of free radicals. Cd interferes with the uptake, transport and use of several elements (Cu, Ni, Zn, Pb, and Cr) and that of water by plants (Das *et al.*, 1997). Cd can be readily taken up and accumulated in vascular plants

(Prasad, 1995, Cataldo *et al.*, 1983, Kawashima *et al.*, 1991). Cd interacts with water balance (Barcelo *et al.*, 1986, Poschenrieder *et al.*, 1989, Costa and Morel, 1994).

Effect of cadmium on growth

The growth of the cell and that of the whole plant is drastically reduced by Cd toxicity. The presence of the heavy metal in the soil, decreases the growth of soybean (Cataldo *et al.*, 1983, Dowdy *et al.*, 1997), pigeon pea (Streaty and Rao,1999).

Effect of cadmium on fresh and dry mass

An increase in the concentration of the cadmium decreases the fresh mass in mung bean (Shen *et al.,* 1998) dry mass in chick pea and green gram (Rana *et al.,* 2002).

Effect of cadmium on nodulation

The presence of heavy metal in the soil decreases the yield of symbiotic nitrogen fixing organisms and the number of nodules per root (Vigue *et al.*, 1981). The presence of Cd decreased the nodulation and nitrogenase activity in *Phaseolus vulgaris* (Dewdy and Ham, 1997; Vigue *et al.*, 1981), *Trifolium repens* (McGrath *et al.*, 1988), soybean (Vespa *et al.*, 1978) and *Alnus rubra* (Wickliff *et al.*, 1995). Nitrogen assimilation in pea plants was severely affected on being exposed to Cd (Hernandez *et al.*, 1995a). A positive correlation was observed between leghemoglobin content and nitrogenase activity (Darkaro, 1995; Comba *et al.*, 1997, 1998) and both

these parameters exhibited a parallel decrease in the presence of Cd (Farnandez *et al.*, 1996).

Effect of cadmium on photosynthesis

Cadmium is an effective inhibitor of photosynthesis (Greger *et al.*, 1994). A linear relationship between photosynthesis and inhibition of transpiration was observed in clover, lucerne, and soybean that suggests Cd inhibited stomatal opening (Barcelo *et al.,* 1990). Cd damages the photosynthetic apparatus, in particular the light harvesting complex II (Krupa, 1988) and the photosystem I and II (Siedlecka *et al.*, 1993, 1996). The inhibition of root FeIII reductase induced by Cd leads to Fe(II) deficiency and that seriously affected photosynthesis (Alkantara *et al.*, 1994). Cd causes stomatal closure in higher plants (Poschenneder *et al.*, 1989) and an overall inhibition of photosynthesis (Sheoran *et al.*, 1990, Krupa *et al.*, 1993, Chaugh *et al.*, 1999).

Effect of cadmium on the activity of nitrate reductase

Nitrate reductase (NR) the primary enzyme in the nitrate assimilation pathway, is the limiting factor in plant growth and development (Salomonson and Barber, 1990) and its level is influenced by a variety of environmental factors (Andrew, 1986; Murphy *et al.*, 1987). The assimilation of NO_3 was influenced by the addition of Cd to the crops of the sugarbeet (Petrovic *et al.*, 1990) maize (Nassbaum *et al.*, 1988; Hernandez *et al.*, 1996) pea (Burzynski, 1998) *Silene vulgaris* (Marthy's, 1975) wheat (Buczek *et al.*, 1980), bean and tomato (Quariti *et al.*, 1997), *Picea alba* (Yandow *et al.*, 1981).

Effect of cadmium on chlorophyll

The presence of Cd decreased the content of chlorophyll, carotenoid and increased the non-photochemical quencing in *Brassica napus* (Larsen *et al.*, 1998). Similarly, the synthesis and the level of chlorophyll decreased in other plant species under the influence of the cadmium (Czuba *et al.*, 1973; Imai *et al.*, 1973; Baszynski *et al.*, 1980; Griffiths *et al.*, 1985; Stiborova *et al.*, 1986; Bishnoi *et al.*, 1993; Ferreli *et al.*, 1994).

Effect of cadmium on protein content

The growth reduction associated with the Cd treatment was probably caused by the inhibition of protein synthesis (Foy *et al.*, 1978). Phytotoxicity of the metal in other crop plants has also been observed in the form of a loss in the level of protein (Dubey and Dwivedi, 1987; Gupta *et al.*, 1995; Tamas *et al.*, 1997). Moreover the grains developed on the plants grown under Cd stress had lower protein content (Salgare and Achareker, 1992).

Effect of cadmium on proline content

Accumulation of free proline in response to heavy metal exposure seems to be wide-spread among plants (Saradhi, 1981; Bassi *et al.*, 1993a,b; Costa *et al.*, 1994). However Cd is the strongest inducer of proline compared with Hg, Pb, Zn and Cu (Saradhi, 1981). In rice (Roy *et al.*, 1992) proline so generated could be involved in metal chelation in cytoplasm (Farago *et al.*, 1979). An increase in the constitutive proline level have been observed in a copper-tolerant ecotype of *Armenia meritima* (Farago, 1981).

Effect of cadmium on enzymes

Cd strongly affects the activity of carbonic anhydrase (Siedlecka *et al.*, 1997). The metal also produces oxidative stress (Hendy *et al.*, 1992; Somashekaraiah *et al.*, 1992). Cd ions can inhibit the activity of several antioxidative enzymes. In *Helianthus annus* leaves, Cd enhanced lipid peroxidation, increased lipoxygenase activity and decreased the activity of the antioxidative enzymes e.g. superoxidase dismutase, catalase, peroxidase glutathione, reductase and dehydroascorbate reductase (Gallego *et al.*, 1996). Peroxidase induction is a general response of higher plants to the uptake of toxic level of the metals (Van *et al.*, 1990). Cd induced peroxidase activity in the root and leaves of *Oryza sativa*. Moreover, the root showed 10-20 fold higher activity of peroxidase than the leaves (Reddy and Prasad, 1990). In *Phaseolus*, the supply of Cd raised the level of lipid peroxidation, guaiacol and ascorbate peroxidation activity (Shaw,1995; Chaoui *et al.*, 1997; Lazano-Rodriguez *et al.*, 1997). However, no peroxidation was noticed in Cd-exposed plants and hairy roots of *Daucus carota* (Sanita-di-Toppi *et al.*, 1998a,b).

Effect of cadmium on yield

Decrease in the yield of red clover has been reported by McGranth (1994) because of the toxic effects of Cd.

Brassinosteroids

Earlier, only five group of hormones (auxin, gibberellins, cytokinins, abscissic acid and ethylene) were designated as regulators of

plant growth and development. However, in the recent past, compelling evidences have been put forward to classify additional group of phytohormones, including steroidal substances (Brassinosteroids).

It was in 1970, when Mitchell and co-workers screened the pollens of nearly sixty species, out of which the extract from about thirty species generated growth in bean seedlings. This growth promoting substance was called "Brassin". The search for its active factor(s) was collectively approached in 1974 by the USDA scientists working at Northern Regional Research Centre (NRRC); Peoria Eastern Regional Research Centre (ERRC), Philadelphia and Beltsville Agricultural Research Centre (BARC) Maryland. Bee-collected pollens (500 lb) were processed through a pilot plant-size solvents (2-propanol) extraction purification at BARC. However, it was crystallized at NRRC and was subjected to X-ray analysis to establish its structure. This biologically active plant growth promoter was found to be steroidal lactone ($C_{28}H_{48}O_6$) and was named as "brassinosteroid".

All natural brassinosteroids have a common 5-α-cholestron skeleton and its structural variants come from the type and the orientation of functionalities on the skeleton. Their distribution in plants is not uniform throughout its body but young growing tissues have comparatively a large share than the mature tissues (Yokata and Takahashi, 1986). The richest sources are pollen and immature seeds where its concentration ranges between 1-100 ng per g (fresh mass) whereas the shoot and the leaves have about 0.01-0.1 ng per g (fresh mass) (Takatsuto, 1994). Till now more than 70 brassinosteroids, structurally and functionally different from each other

have been characterized (Bajguz and Treytn, 2003). Out of which, three (brassinolide, 24-epibrassinolide and 28-homobrassinolide) are being largely applied to have an economical impact on plant metabolism, growth and productivity.

Occurrence of brassinosteroids

About 70 BRs (65-unconjugated and 5-conjugated) have so far been isolated from 60 plant species, including 51 angiosperms (12 monocotyledons and 39-dictoyledons), 6 gymnosperms, 1 pteridophyta (*Equisetum arvense*) 1 bryophyta (*Marchantia polymorpha*) and 1 chlorophyta, the alga (*Hydrodictyon reticulatum*). Thus BRs are widely distributed in all organs of both higher and lower plants (Bajguz and Tretyn, 2003). The gall of *Castanea crenata* and *Distylium racemosum* have higher levels of BRs (several μg/kg) than the normal, healthy tissues. Similarly crown gall cells of *Catharanthus roseus* have higher contents of brassinolide (BL) and Castasterone (CS) (30-40 μg/kg) than the normal cells. The highest concentration of BR 6.4 mg per 1 kg pollen, was detected in *Cupressus arizonica* (Griffiths *et al.*, 1995; Clouse and Sasse, 1998; Fujioka, 1999).

Among the BRs, CS is the most widely distributed (50 plant species), followed by BL (34 plant species), TY (25 plant species), 6-deoxo CS (19 plant species), TE (19 plant species) and 28-nor CS (12 plant species). To the present day 34-other BRs and 5 BR conjugates have been found in only one plant species. Among all naturally occurring BRs. CS and BL are the most important BRs because of their wide distribution as well as

their potent biological activity (Kim, 1991; Fujioka, 1999). Among the plant sources investigated, immature seeds of *Phaseolus vulgaris* contain a wide arrays of BRs (25 free and 2 conjugates). The wide occurrence of BRs was also reported in dwarf mutant of *Catharanthus roseus* (19 compounds) *Arabidopsis thaliana* (18 compounds), *Cyrptomeria japanica* and *Cupressus arizonica* (9 compounds), *Dolichos lablab*, *Oryza sativa, Thea sinensis* and *Scecela cereals* (8 compounds), *Lilium longiflorum* (7 compounds). *Distylium racemosum* (6 compounds) (Bajguz and Tretyr, 2003).

Effect of brassinosteoids on seed germination

Endogenous BRs have been identified in the seeds of several species, including pea (Yokata *et al.*, 1996), *A. thaliana* (Schmidt *et al.*, 1997) and *Lychnis viscaria* (Friebe *et al.*, 1999). It is well documented that brassinosteroids promote seed germination, like other hormones. The treatment of the seeds of *Lepidium sativus* (Jones-Held *et al.*, 1996) and *Eucalyptus camaldulensis* (Sasse *et al.*, 1995) with brassinolide improved germination. Similarly, brassiosteroids promote seed germination in case of *Brassica napus* (Chang and Cai, 1998), rice (Dong *et al.*, 1989), wheat (Sairam *et al.*, 1996; Hayat *et al.*, 2003), tomato (Vardhini and Rao, 2000) and tobacco (Lenbner-Metzger, 2001). Moreover, brassinolide, 24-epibrassinolide and 28-homobrassinolide, promoted seed germination in groundnut (Vardhini and Rao, 1997). BR application has been reported to enhance the germination of certain parasitic angiosperms (Takeuchi *et al.*, 1991, 1995) cereals (Gregory, 1981; Yamaguchi *et al.*, 1987). The treatment

of barley grain also accelerated subsequent seedling growth (Gregory, 1981).

In *Arabidopsis thaliana*, BR promotes the germination of pre-chilled (i.e. non-dormant) seeds of BR-deficient biosynthesis mutant det2-1 and the BR-insensitive response mutant bri1-1-imbibed in the light (Steber and McCourt, 2001). Seed germination of det2-1 and bri1-1 is more strongly inhibited by ABA than the wild type and BR is able to partially overcome the inhibition of germination by ABA. BR treatment rescues the germination in phenotype of the severe GA-deficient biosynthesis mutant gai-3, which normally requires GA treatment for dormancy release and germination. BR treatment also partially rescues the germination in phenotype of the severe GA-insensitive response mutant Sly-1 (Sleepy 1), which cannot be rescued by treatment with GA. Interestingly, a new allel for sly 1 was identified in a screen for BR-dependent germination and is also proposed to be based on interaction between BR and GA signelling in seeds (Steber *et al.*, 1998; Steber and McCourt, 2001). This is further supported by the germination phenotype of the gpa1 mutant of *Arabidopsis* (Ullah *et al.*, 2002). BR promotes seedling elongation and germination of non-photodormant tobacco seeds, but do not appreciably affect testa rupture and subsequent induction of b-Glu1 in the micropylar endosperm (Leubner-Metzger, 2001, 2003). Treatment with BR accelerates endosperm rupture in tobacco seeds, imbibed in the light. Promotion of endosperm rupture by BR is dose-dependent and 0.01M brassinolide is most effective (Leubner-Metzger, 2003).

Effect of brassinosteroids on fresh and dry mass of plants

The foliage application of 28-HBR increased plant fresh and dry mass in mustard (Hayat *et al.*, 2000, 2001) and mung bean (Fariduddin *et al.*, 2004, 2005). Similarly, 24-epibrassinolids (EBR), applied to the foliage enhanced the plant mass, root and shoot length, their fresh and dry mass in chickpea (Singh *et al.*, 1993) and groundnut (Vardhini and Rao, 1997). The seedlings raised from the grains pre-treated with HBR possessed significantly higher fresh and dry mass plant^{-1} (Hayat *et al.*, 2001).

Effect of brassinosteroids on nodulation and nitrogenase activity

BRs application to the foliage increases the number of nodules, fresh mass of nodulated roots and nitrogenase activity in *Arachis hypogea* (Vardhini and Rao, 1997) and *Cicer arietinum* (Ali *et al.*, 2006). However, a decrease in the root length and nodule number was noted in *Lens culinaris* plants, raised from the seeds given pre-sowing soaking treatment with 28 homobrassinolide (Hayat and Ahmad, 2003).

Effect of brassinosteroids on flowering

There has been a limited application of steroids in regulating flowering. The number of flowers in strawberry increased by the application of brassinosteroids to their foliage (Pipettanawong *et al.*, 1996). However, in case of grapes, the application of brassinosteroids in autumn improved the number of flowers but inhibited if the time of application is delayed to late winter (Rao *et al.*, 2002).

Effect of brassinosteroids on senescence

It is the process, which refers to endogenously regulated deteriorative changes that become the natural cause of death of cells, tissue, organ or that of the whole organism (Arteca, 1997). Like other hormones, brassinosteroids also play a crucial role in regulating the process leading to senescence (Rao *et al.*, 2002). The brassinolide promotes senescence in *Xanthinum* and *Rumex* explants (Mandava *et al.,* 1981). In addition to it, brassinosteroids also accelerate senescence in the detached cotylendons of cucumber seedlings (Zhao *et al.*, 1990) and to the leaves of mung bean seedlings (He *et al.*, 1996). However, brassinosteroids deficient *Arabidopsis* mutant exhibited delayed senescence of chloroplast (Li *et al.,* 1996). Similarly, the senescence of the leaves of mung bean and mustard was delayed, if supplied with 28-homobrassinolide, at early stage of growth (Fariduddin, 2002). During a search of senescence regulating network in *Arabidopsis*, where signals such as abscissic acid, jasmonic acid, ethylene, darkness, dehydration and aging activated 147 senescence associated enhancer trap line, 24-epibrassinolide could activate some of these but the associated genes could not be cloned.

Effect of brassinosteroids on photosynthesis

The aqueous solution of 28-homobrassinolide, applied to the foliage of wheat and mustard (Sairam 1994; Hayat *et al.*, 2000; 2001 a) or as pre-sowing seed soaking treatment to mung bean (Fariduddin *et al.,* 2003, 2004a) and dialkylaminoethylalkanote or epibrassinolide in association with GA_3 to spinach (Liang *et al.,* 1998) enhanced the photosynthetic rate. Foliage spray of aqueous solution of BR to wheat and mustard (Braun and Wild, 1984), and

brassinolide to rice (Fujii *et al.,* 1991) increased the rate of CO_2 assimilation. Likewise, the foliar application of 24-epibrassinolide enhanced the light saturated net CO_2-assimilation rate and carboxylation rate of rubisco, there by increasing the capacity of CO_2 assimilation in the Calvin cycle (Yu *et al.*, 2004). However, the epicotyl of cucumber, did not respond to epibrassinolide but the transport of the labeled (C^{14}) glucose towards the epicotyl was favoured (Nakajima and Tayama, 1995). Similarly, Hill activity in the foliage of *Vigna radiata* was favourably affected, on being supplemented with aqueous solution of 28-homobrassinolide (Bhatia and Kaur, 1997).

Effect of brassinosteroids on chlorophyll content

The total chlorophyll content or its fractions increased in the leaves of wheat and mustard (Braun and Wild, 1984) maize (Shen *et al.*, 1990) *Vigna radiata* (Bhatia and Kaur, 1997) and *Brassica juncea* (Hayat *et al.*, 2001a) by 28-homobrassinolide and in *Cucumis sativus* (Yu *et al.*, 2004) by epibrassinolide, applied directly to their foliage. Similarly, the values for the above parameter increased in the leaves of rice (Wang, 1997), *Cicer arietinum* (Fariduddin *et al.,* 2000), *Brassica juncea (*Hayat and Ahmad, 2003b*)* and *Vigna radiata* (Fariduddin *et al.,* 2003) raised from the seeds given pre-sowing treatment with BRs/28-homobrassinolide. Moreover, the water stressed plants, treated with 28-homobrassinolide, possessed high chlorophyll level (Sairam, 1994).

Effect of brassinosteroids on carbonic anhydrase activity

Carbonic anhydrase (CA), is the second most abundant soluble protein, other than RuBPase in C_3-chloroplast (Reed and Graham, 1981; Okabe

et al., 1984). It is a zinc containing protein with a molecular weight of 180 Kda (Lawlor, 1987) and is a ubiquitous enzyme, among living organism. It catalyzes the reversible inter conversion of bicarbonates (HCO_3^-) and CO_2 (Sultemeyer *et al.*, 1993). The rate of conversion of HCO_3^- to CO_2 is normally slow in alkaline conditions. However, CA activates the use of HCO_3^- in the production of CO_2 (Lawlor, 1987). In C_3 plants, CA has a close association with RuBPase where it elevates the level of CO_2 at its active site (Badger and Price, 1994). An increase in the activity of CA, in the leaves, was attained by the application of 28-homobrassinolide to the shoot of the *Brassica juncea* (Hayat *et al.*, 2000, 2001a, 2003). Moreover, the seedlings of wheat and mungbean, raised from the grains treated with 28-homobrassinolide, possessed high CA activity in their leaves (Hayat *et al.*, 2001b; Fariduddin *et al.*, 2003, 2004).

Effect of brassinosteroids on nitrate reductase activity

The process of reduction of nitrate is catalysed by the enzymes, nitrate reductase (NR). The level of NR increased in the plants of rice (Mai *et al.*, 1989), maize (Shen *et al.*, 1990), water stressed wheat (Sairam, 1994), *Lens culinaris* (Hayat and Ahmad, 2003a,b), *Vigna radiata* (Fariduddin *et al.*, 2004b) and wheat (Hayat *et al.*, 2001b) and in the seeds of wheat (Hayat and Ahmad, 2003c) by the application of BRs.

Effect of brassinosteroids on the vascular tissue

The very first report of the involvement of BRs in the differentiation of vascular tissues appeared in 1991 (Clause and Zurek, 1991). The cells of *Helianthus tuberoses* transferred to a medium, in the presence of auxin and

cytokinin differentiated into xylem elements in 72-96 hours. Very few vascular elements develop in the first 24-hours following transfer into this medium. However, non-molar concentrations of BL included in the medium, resulted in a 10-fold increase in xylem differentiation, this was observed in the first 24 hours. Moreover, the cell number increased significantly, indicating a role for BRs in cell division and differentiation.

Zinnia elegans has been used extensively to study the formation of xylem/trachery elements, a process that has three distinct stages (Fukuda, 1997). BRs have been implicated in the transition between stages II and stages III where secondary wall formation and cell death occurs. It has been observed earlier that uniconazole (a putative BR biosynthesis inhibitor) prevents differentiation of *Zinnia* mesophyll cells into trachery elements and this inhibition was overcome by exogenous BR application (Iwasaki and Shibaoka, 1991). Uniconazole appears to suppress the transcription of genes involved in the final stages of differentiation but could be recovered by the exogenous application of BL (Yamamoto *et al.*, 1997). This suggests that BRs are synthesized prior to secondary cell wall development and cell death and possibly induces the entry into this stage (Yomamato *et al.*, 1997).

Effect of brassinosteroids on stressed plants

The resistance to abiotic stresses is increased in the plants, treated with brassinosteroids. The plants of tomato and rice (Kamuro and Takatsuto, 1991) maize (He *et al.*, 1991), cucumber (Katsumi, 1991) wheat (Kulaeva *et al.*, 1991) and brome grass (Wilen *et al.*, 1995), developed an elevated level of resistance to low or high temperature, on being treated with brassinosteroids.

Similarly, in chilling stressed, cucumber plants, pre treatment with 24-epibrassinolide or ABA improved tolerance and efficiency of photosystem II (Yu *et al.*, 2002). Application of 28-homobrassinolide increased tolerance to water stress in sugarbeet (Schilling *et al.*, 1991) and wheat (Sairam, 1994).

The ability of brassinosteroids to counteract the inhibitory, effect of salinity on seedling growth of groundnut (Vardhini and Rao, 1997) and seed germination in rice (Anuradha and Rao, 2001) and *Eucalyptus camaldulensis* (Sasse *et al.*, 1995) was reported. Similarly, 28-homobrassinolide improved growth nodulation, nitrogen fixation and overall metabolic activities in *Cicer arietinum*, under NaCl stress (Ali *et al.*, 2006). Analysing the effect of BB-16 (a polyhydroxylated spirostanic brassinosteroid analogue) on the rice seedlings, grown in culture medium supplemented with NaCl, Nanez *et al.* (2003) found that it causes a significant increase in the activity of antioxidative enzymes (viz. catalase, superoxide dismutase, glutathione reductase and ascorbate peroxidase). Likewise, Kamuro and Takatsuto (1999), were also impressed by the ability of brassinosteroids that confer resistance in plants, against a wide variety of environmental stresses. They were of the opinion that the role of brassinosteroids in protecting the plants against environmental stresses will be an important research theme and may contribute largely to the usage of brassinosteroids in agricultural production.

Effect of brassinosteroids on the yield of crops

Once the presence of brassinosteroids in plants was established the next phase was to explore the possibility of their involvement in improving the biological yield of economically useful plants. Brassinolide was first employed

to increase the yield of lettuce, reddish, bush bean and pepper (Meudt *et al.*, 1983, 1984). Moreover, dilute aqueous solution of brassinolide improved the yield of wheat and mustard (Braun and Wild, 1984), rice, corn and tobacco (Yokota and Takahashi,1986) on being applied to the foliage. Similarly, brassinosteroids also increased the growth and yield of sugarbeet (Schilling *et al.*, 1991) legumes (Kamuro and Takatsuto, 1992) and rapeseed (Takematsu and Takeuchi, 1989, Hayat *et al.*, 2000; 2001b). Application of 28-homobrassinolide and 24-epibrassinolide significantly increased yield of potato, mustard, rice and cotton (Ramraj *et al.*, 1997), *Lens culinaris* (Hayat and Ahmad, 2003a,b), *Vigna radiata* (Fariduddin *et al.*, 2003) mustard (Hayat *et al.*, 2000, 2001) and that of corn, tobacco, watermelon, cucumber and grape (Ikekawa and Zhao, 1991). Their utility was further proved by the foliar application of 24-epibrassinolide and 28-homobrassinolide in enhancing the yield of groundnut and tomato (Vardhini and Rao, 1997, 1998, 2001). Moreover, in China 28-homobrassinolide has been registered as a plant growth regulator in case of tobacco, sugarcane, rapeseed and tea.

Materials and Methods

CHAPTER 3

MATERIAL AND METHODS

Proposed Study

To achieve the objective framed in chapter 1, *Cicer arietinum* L. cv. Uday was grown in soil by generating stress with cadmium chloride.

Seeds

The seeds of *Cicer arietinum* L. cv. Uday were purchased from national Seed Corporation Ltd. Pusa New Delhi. Healthy seeds were surface sterilized with 0.01% aqueous solution of mercuric chloride ($HgCl_2$) followed by repeated washing with double distilled water (DDW).

Hormone preparation

28-homobrassinolide (HBR) was obtained from Godrej Agrovet Ltd., Mumbai. 10^{-4}M stock solution was prepared by disso lving required quantity of the hormone in 5 cm^3 of ethanol, in a 100 cm^3 volumetric flasks. 5 cm^3 surfactant "Tween-20" was added to it and final volume was made upto the mark by using DDW. The desired concentration of HBR were prepared by the dilution of stock solution.

Experiments

The experiment was conducted with 80 pots in such a way that each treatment have 10 pots (replicate) and with in each pot there are four plants, under complete randomized design technique, to study the effect of HBR on cadmium induced changes in chickpea in the following manner.

The surface sterilized seeds were inoculated with specific *Rhizobium* and the sowing was done in earthen pots (10 inch diameter), filled with sandy loam soil and farmyard manure (mixed in the ratio of 6:1) and allowed to germinate for 15 days.

The pots holding 15 days old seedlings were supplied with 0, 50, 100, 150 μM cadmium, in the form of CdCl through roots. At 30 day stage, plants were sprayed with 0 or 10^{-8}M HBR. Each seedling was sprinkled thrice. The nozel of the sprayer was adjusted in such a way that it pumped out 1 cm^3 in one sprinkle. Therefore, each plant received 3 cm^3 of DDW or HBR solution.

The plants were sampled at 60 and 90 days, after sowing (DAS) to make the following observations.

1. Plant fresh mass
2. Plant dry mass
3. Nodule number
4. Nodule fresh and dry mass
5. Nitrogenase activity
6. Leghemoglobin content, in fresh nodules
7. Nodule nitrogen content
8. Nodule carbohydrate content
9. Nitrate reductase activity in leaves
10. Carbonic anhydrase activity in leaves
11. Leaf chlorophyll content
12. Leaf proline content

13. Peroxidase activity
14. Catalyse activity
15. Superoxide dismutase activity
16. Seed protein content

The remaining plants in 5-pots each having 4 plants were allowed to grow to maturity to study the following characteristics, at harvest (160 DAS).

1. Number of pods per plant
2. Number of seeds per pod
3. Seed yield per plant
4. 100 seed weight
5. Seed protein content

Dry mass of plant

The plants were uprooted and washed under running tap water and dried in hot air oven, run at 80°C, for 24 hours. The samples were weighed to obtain dry mass.

Nodule number per plant

The whole mass of the soil was taken out of the pot and placed in bucket, filled with water. The plants were moved to uproot them, with no damage to the nodules. The roots were washed, under running tap water, and the number of nodules was counted.

Nodule fresh and dry mass

The nodules from each plant were picked and weighed to get their fresh mass. The nodules were then transferred to petriplates for overnight drying in an oven run at 80°C. This dried material was weighed to obtain dry mass of nodules per plant.

Nitrogenase activity in nodules

The nitrogenase activity was assayed by adopting the procedure of Hardy *et al.* (1968). Nodulated roots were cut and shaken slowly to remove the adhering soil particles. Samples were assayed in 30 cm^3 glass tubes, sealed with a subseal to allow it to be pierced by an hypodermic needle, bearing a syringe. 10% (v/v) of air was withdrawn from the sample container and replaced by an equal volume of acetylene gas. After 1 hour of incubation, at room temperature 0.5 cm^3 of gas was injected into a gas chromatograph (Nucon Series 5500, New Delhi) equipped with a flame ionization detector to quantify the ethylene produced. The result were expressed in terms of nano moles of ethylene formed/g (nodule fresh mass)/hour.

Leghemoglobin content

The leghemoglobin content, in fresh nodules, was estimated following the method described by Sadasivam and Mannickam (1992).

200 mg nodules were mixed with 3 cm^3 of 0.1M phosphate buffer (Appendix 1.1) and macerated in a mixer, followed by filtration through two

layers of cheese cloth. The nodule debris were discarded. The turbid reddish brown filtrate was centrifuged at 10,000 g for 10-30 minutes.

3 cm^3 of pyridine reagent (Appendix 1.2) was added to 3 cm^3 of extract with mixing. The solution become greenish yellow due to the formation of hemochrome.

The hemochrome was divided equally into two test tubes. To one test tube a 50 mg crystals of potassium hexacynoferrate were added to oxidize the hemochrome and read at 539 nm on spectrophotometer (Spectronic 20D, Milton Roy, USA). To the other test tube a 50 mg crystals of sodium dithionate were added to reduce the hemochrome. This mixture was read at 556 nm after an interval of 5 minutes, against a reagent blank.

The leghemoglobin content (mM) was calculated by using the formula:

$$\text{Lb concentration (mM)} = \frac{A_{556} - A_{539}}{23.4} \times 2D$$

where D is initial dilution; A_{556} and A_{539} are absorbance at 556 and 539 nm, respectively.

Nodule nitrogen content

The nodule nitrogen content was estimated by employing the method of Lindner (1944).

Digestion of nodule power

50 mg of the oven dried powder was transferred to a digestion tube to which 2 cm^3 sulphuric acid (AR grade) was added. The digestion tube

was heated on temperature controlled digestion assembly for 2h to allow the complete reduction of nitrogen present in the material. After cooling the digestion tube for about 15 minutes 0.5 cm^3 of 30% H_2O_2 was added drop by drop and the solution was heated again until the colour turned to light yellow. After cooling for 30 minutes, an additional 3 to 4 drops of 30% H_2O_2 were added followed by heating for about 15 minutes. The process was repeated till the contents of digestion tube turned colourless. This digested material was transferred to a 50 cm^3 volumetric flask after 3 washings. The final volume was made upto the mark by using DDW.

Estimation of nitrogen

10 cm^3 of this digested aliquot was taken in a 50 cm^3 volumetric flask and neutralized by adding 2 cm^3 of 2.5N NaOH (Appendix 2.1) and 1% sodium silicate (Appendix 2.2). Volume was made upto the mark by using DDW. 5 cm^3 of this sample was pipetted into a graduated test tube to which 0.5 cm^3 Nessler's reagent was added dropwise, with repeated shaking. The final volume was made upto 10 cm^3 with DDW. After waiting for 5 minutes to get optimum colour development, absorbance of solution was read at 525 nm on spectrophotometer (Spectronic 20D, Milton Roy, USA). A blank consisting of Nessler's reagent and DDW was run simultaneously with each set of samples. Standard curve was plotted by using known, graded dilutions of ammonium sulphate solution. The absorbance of each sample was compared with that of the calibration curve and per cent nitrogen, in each sample, was computed on dry mass basis.

Estimation of total carbohydrate content in nodules

The carbohydrate was extracted from the sample, following the method of Yih and Clark (1965) and estimated by adopting the procedure of Dubois *et al.* (1956).

50 mg of dried nodule powder was transferred to a glass centrifuge tube containing 5 cm^3 of 1.5N H_2SO_4 (Appendix 3.1). The sample was centrifuged at 4000 rpm for 10 minutes. The supernatant was decanted into a 25 cm^3 volumetric flask with two washings of residue with DDW. The volume was made upto the mark using DDW. 1 cm^3 of this extract was taken in a test tube to which 1 cm^3 of 5% distilled phenol (Appendix 3.2) was added. The test tube was placed in chilled water and 5 cm^3 of concentrated H_2SO_4 (AR) was added. The absorbance was read at 490 nm on spectrophotometer. A blank was run simultaneously with each set of samples. Standard curve was plotted by using known graded dilutions of glucose solution. The absorbance of each sample was compared with calibration curve and per cent carbohydrate content was calculated on dry mass basis.

Nitrate reductase (NR) activity

The activity of nitrate reductase was measured following the method laid down by Jaworski (1971), in fresh leaf samples.

The leaves were cut into small pieces (1 cm^2). 200 mg of these chopped leaves was weighed and transferred to plastic vials. To each vial 2.5 cm^3 of phosphate buffer pH 7.5 (Appendix 4.1) and 0.5 cm^3 of

potassium nitrate solution (Appendix 4.2) was added followed by the addition of 2.5 cm^3 of 5% isopropanol (Appendix 4.3). These vials were incubated in BOD incubator for 2h at 30±2°C, in dark. 0.4 cm^3 of incubated mixture was taken in a test tube to which 0.3 cm^3 each of sulphanilamide solution (Appendix 4.4) and NED-HCl (Appendix 4.5) were added. The test tube was left for 20 minutes, for maximum colour development. The mixture was diluted to 5 cm^3 with DDW. The absorbance was read at 540 nm on spectrophotometer. A blank was run simultaneously with each sample. Standard curve was plotted by using known graded concentration of $NaNO_2$ (sodium nitrite) solution. The absorbance of each sample was compared with that of the calibration curve and nitrate reductase activity (nM $g^{-1}h^{-1}$) was computed on fresh mass basis.

Carbonic anhydrase (CA) activity

The carbonic anhydrase activity in the leaves was measured by following the method described by Dwivedi and Randhava (1974).

The fresh leaf samples were cut into small pieces at a temperature below 25°C. 200 mg of these pieces were weighed and transferred to petriplates. The leaf pieces were cut further into smaller pieces in 10 cm^3 of 0.2M cystein hydrochloride (Appendix 5.1) and left at 4°C for 20 minutes. The leaf pieces were blotted and transferred to a test tube containing 4 cm^3 of phosphate buffer of pH 6.8 (Appendix 5.2). To this test tube 4 cm^3 of 0.2M sodium bicarbonate (Appendix 5.3) solution and 0.2 cm^3 of 0.002% bromothymol blue (Appendix 5.4) were added. The test tube was shaken gently and left at 4°C for 20 minutes. CO_2 liberated by the catalytic action of

CA on $NaHCO_3$ was estimated by titrating the reaction mixture against 0.05N HCl (Appendix 5.5) using methyl red (Appendix 5.6) as indicator. In each sample the quantity of HCl used to neutrilize reaction mixture was noted and difference was calculated. A blank consisting of all the above components of reaction mixture, except the leaf sample, was run simultaneously with each set of samples. The activity of the enzyme was calculated by putting the values in the formula.

$$\frac{V \times 22 \times N}{W} [Mol\ (CO_2)kg^{-1}(leaf.F.M.)S^{-1}]$$

Where, V = difference in volume (cm^3 of HCl used in control and test sample titration)

22 = equivalent weight of CO_2

N = Normality of HCl

W = Fresh mass of tissue used

Chlorophyll content

The chlorophyll content in the fresh leaf was estimated following the method worked out by Mackinney (1941).

1g of finely cut fresh leaves was ground to a fine pulp using a mortar and pestle after pouring 20 cm^3 of 80% acetone. The mixture was centrifuged at 5,000 rpm, for 5 minutes. The supernatant was collected in 100 cm^3 volumetric flask. The residue was washed three times, using 80% acetone (Appendix 6). Each washing was collected in the same volumetric flask and volume was made upto the mark, using 80% acetone. The absorbance was read at 645 and 663 nm against the (80% acetone) blank on

spectrophotometer. The chlorophyll content present in the extract (mg g^{-1} tissue) was calculated using the following equation

$$\text{mg chlorophyll per kg tissue} = 12.7\,(A_{663}) - 2.69\,(A_{645}) \times \frac{V}{1000xW}$$

$$\text{mg chlorophyll per kg tissue} = 22.9\,(A_{645}) - 4.68\,(A_{663}) \times \frac{V}{1000xW}$$

A = absorbance at specific wavelengths

V = final volume of chlorophyll extract in 80% acetone

W = fresh mass of tissue, used for extraction

Proline content

The proline content in fresh leaves was estimated following the procedure used by Bates *et al.* (1973). 0.5 g of fresh leaf sample was homogenized in a mortar with 5 cm^3 of 3% sulphosalicylic acid (Appendix 7.1). The homogenate was filtered through Whatman filter paper No. 2 and collected in a test tube with two washings each with 5 cm^3 of sulphosalicyclic acid. 2 cm^3 each of glacial acetic acid and acid ninhydrin (Appendix 7.2) was added to 2 cm^3 of the above extract. This mixture was heated in boiling water for 1h. The reaction was terminated by transferring the test tubes to ice-bath. 4 cm^3 of toluene was mixed to the reaction mixture with vigorously shaking, for 20-30 seconds. The chromophore (toluene) layer was aspirated and warmed to room temperature. The absorbance of red colour was read at 520 nm against a reagent blank. The amount of proline in the sample was calculated by using a standard curve prepared from pure

proline (range 0.1-36 μ mol) and expressed on fresh mass basis of the sample.

$$\mu \text{ moles of proline } g^{-1} \text{ tissue} = \frac{\mu \text{ g proline } cm^{-3} \text{ x } cm^{-3} \text{ toluene}}{115.5} \text{ x } \frac{5}{\text{g (sample)}}$$

where 115.5 is the molecular mass of the proline.

Estimation of peroxidase, catalase and superoxide dismutase

500 mg of leaf tissue was homogenized in 5 cm^3 of 50 mM phosphate buffer (pH 7.0) containing 1% polyvinyl pyrilidone. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 5°C and the supernatant obtained was used as extract for peroxidase, catalase and superoxide dismutase.

Estimation of peroxidase

3 cm^3 of pyragallol phosphate buffer (Appendix 8.1), 0.1 cm^3 of enzyme extract and 0.5 cm^3 of 1% H_2O_2 were mixed in a cuvette and a change in absorbance, at 20 seconds interval for a period of 3 minutes was read at 420 nm on a spectrophotometer. The control set was prepared by boiling the enzyme extract (Chance and Machly, 1956).

Estimation of catalase

The estimation of catalase was done by permagnate titration method (Chance and Machly, 1956). 3 cm^3 of phosphate buffer (pH 6.8) (Appendix 9.1). 1 cm^3 of 0.1M H_2O_2 (Appendix 9.2) and 1 cm^3 of enzyme extract were mixed and this mixture was incubated at 25°C for 1 minute. Then 10 cm^3 of 2% H_2SO_4 (Appendix 9.3) was added. The mixture was titrated against 0.1N

potassium permagnate (Appendix 9.4) to find the residual H_2O_2 until a purple colour persists for at least 15 sec. Similarly, a control set was maintained in which the enzyme activity was stopped by the addition of H_2SO_4, prior to the addition of enzyme extract.

Estimation of superoxide dismutase (SOD)

The activity of SOD was measured by the method of Beauchamp and Fridovich (1971). A 3 cm^3 of reaction mixture containing 1 ml of 50 mM phosphate buffer (pH 7.8) (Appendix 10.1). 0.5 cm^3 of 13 mM methionine (Appendix 10.2), 0.5 ml of 75 mM NBT (Appendix 10.3), 0.5 cm^3 of 2 mM (Appendix 10.4), riboflavin, 0.5 cm^3 of 0.1 mM EDTA (Appendix 10.5) and 0.1 cm^3 of enzyme extract was made. Riboflavin was added in the last. The absorbance of the reaction mixture was read at 560 nm on a spectrophotometer.

Protein content

The total protein content in leaves and seeds was estimated by adopting the methodology of Lowry *et al.* (1951).

50 mg of the oven dried powder was transferred to a mortar. The sample was ground with the addition of 1 cm^3 of 5% trichloroacetic acid (Appendix 11.1). The pulp was transferred to a glass centrifuge tube with repeated washings with 5% TCA to make the final volume 5 cm^3. The mixture was centrifuged at 4,000 rpm for 15 minutes and the supernatant was discarded. 5 cm^3 of 1N NaOH (Appendix 11.2) was added to the residue. The tube was left in a water bath at 60°C for 30 minutes. After

cooling for 15 minutes, the mixture was centrifuged at 4,000 rpm for 15 minutes. The supernatant was collected in 25 cm^3 volumetric flask with repeated washing. Volume was made upto the mark by using 1N NaOH and used to estimated total protein content.

1 cm^3 of above extract was transferred to a test tube and 5 cm^3 of reagent C (Appendix 11.3) was added rapidly with immediate mixing. The absorbance of their blue colour was read at 660 nm, using spectrophotometer. A blank was run with each set of samples. The total protein content was calculated by comparing the absorbance of each sample with a calibration curve plotted by taking known graded concentrations of bovin albumin.

Number of pods per plant

At harvest (160 days after sowing), 9 plants (3 from each replicate) from each treatment were randomly sampled and counted for the number of pods per plant.

Number of seeds per pod

25 pods from each treatment were randomly selected for computing the number of seeds per pod.

Seeds yield per plant

The pods from four plants, representing each treatment, were crushed, cleaned to assess the seed weight per plant.

Statistical analysis

The values for various parameters of the plants were subjected to statistical analysis by following the standard procedure described by Gomez and Gomez (1984). The 'F' test was applied to assess the significance of the treatment, at 5% level of probability. Critical difference (CD) among the treatment, was calculated by putting the values of various components in the following formula.

$$CD = \sqrt{\frac{\text{Standard Error x 2}}{\text{Replicates}}} \text{ x t (value) 5\%}$$

Results

CHAPTER 4

RESULTS

Growth characteristics

The growth (fresh and dry mass) of plants increased as the growth progressed from 60 to 90 days (Table 1). The supply of the cadmium decreased the values for both the parameters in proportion to the concentration of the metal. However, the treatment with 10^{-8}M of 28-homobrassinolide (HBR) significantly increased the fresh and dry mass at both the stages of growth, compared with the control. Moreover, HBR (10^{-8}M) also overcame the damaging effect of the cadmium, completely at its lower concentration (50 μM) and partially at higher concentrations (100 and 150 μM).

Nodule number per plant

The values for nodule number increased as the growth proceeds (Table 2). The application of cadmium resulted in a significant loss in their number and that was in proportion to the concentration of the metal. A significant increase in the nodule number was recorded in the plants supplemented with HBR (10^{-8}M). However, the damage caused by the metal was partially overcome if the metal treatment was followed with that of HBR. The values were comparable with that of the control in the case where lower concentration of the metal (50 μM) was associated with HBR.

Nodule Fresh and Dry Mass

These parameters followed a pattern comparable to that of the nodule number (Table 2) in the sense that the values increased with growth

Table 1. Effect of 28-homobrassinolide (HBR) on cadmium (50, 100, 150 μM) induced changes in fresh and dry mass (g) per plant of *Cicer arietinum* at 60 and 90 days, after sowing (DAS).

Treatment	Fresh mass (g)		Dry mass (g)	
	60 DAS	90 DAS	60 DAS	90 DAS
Control	6.16	8.15	1.23	1.69
HBR (10^{-8}M)	7.50	9.95	1.66	2.31
Cd (50 μM)	5.06	7.40	0.97	1.48
Cd (100 μM)	4.60	6.55	0.83	1.23
Cd (150 μM)	3.30	5.48	0.56	0.99
Cd (50 μM)+ HBR (10^{-8}M)	6.72	9.12	1.19	1.98
Cd (100 μM) + HBR (10^{-8}M)	5.32	7.80	0.98	1.50
Cd (150 μM) + HBR (10^{-8}M)	3.70	6.25	0.66	1.31
LSD	0.56	0.69	0.20	0.31

Table 2. Effect of 28-homobrassinolide (HBR) on cadmium (50, 100, 150 μM) induced changes in nodule number and their fresh and dry mass (mg) per plant in *Cicer arietinum* at 60 and 90 days, after sowing (DAS).

Treatment	Nodule number		Nodule fresh mass (mg)		Nodule dry mass (mg)	
	60 DAS	90 DAS	60 DAS	90 DAS	60 DAS	90 DAS
Control	46.6	50.6	310	361	77.5	103.14
HBR (10^{-8}M)	54.3	59.3	370	452	123.3	145.86
Cd (50 μM)	38.0	45.6	261	295	62.14	81.94
Cd (100 μM)	32.3	36.0	213	257	47.33	64.25
Cd (150 μM)	25.0	28.3	148	178	29.60	39.55
Cd (50 μM)+ HBR (10^{-8}M)	49.6	54.6	325	383	58.75	80.00
Cd (100 μM) + HBR (10^{-8}M)	42.3	45.6	280	344	41.16	61.42
Cd (150 μM) + HBR (10^{-8}M)	31.3	35.3	161	193	30.65	51.09
LSD	3.5	4.6	33	39	20.56	26.45

(60 to 90 days) and the supply of cadmium declines them with their increasing concentration (50, 100, 150 μM). However, the supply of HBR alone or as a follow up treatment improved the fresh and dry mass of the nodules and also neutralized the damaging effect of the metal to a limited extent.

Nodule nitrogenase activity

The activity of nitrogenase in the nodules increased from day 60 to 90 (Table 3). The values decreased as the concentration of the cadmium was increased in the solution, supplied to the plants on day 15. However, 10^{-8}M of HBR, applied to the roots, significantly enhanced the activity at day 60 and was maintained at a higher level in the next sampling (90 DAS). The follow up treatment of the metal to the plants with HBR neutralized the damaging effect of the metal to a large extent in this parameter than the earlier ones. Here the effect of even that of 100 μm of the metal was completely over come by HBR.

Leghemoglobin content

Leghemoglobin content exhibited an increase with growth (Table 3). The application of cadmium had a negative impact and the degree of damage depended on the concentration of the metal. However, as in other parameters, HBR increased the values for leghemoglobin content, over that of the control and also completely overcame the ill effect generated by 50 μM of cadmium but partially that of higher concentrations (100 and 150 μM).

Table 3. Effect of 28-homobrassinolide (HBR) on cadmium (50, 100, 150 μM) induced changes on nitrogenase activity [n mol C_2H_5 (g nodule FM)$^{-1}h^{-1}$] and leghemoglobin [m mol(g F.M)$^{-1}$] in *Cicer arietinum* at 60 and 90 days, after sowing (DAS).

Treatment	Nodule nitrogenase acxtivity		Leghemoglobin	
	60 DAS	90 DAS	60 DAS	90 DAS
Control	388	405	34	46
HBR (10^{-8}M)	439	462	55	74
Cd (50 μM)	371	390	28	42
Cd (100 μM)	352	367	21	30
Cd (150 μM)	334	345	13	25
Cd (50 μM)+ HBR (10^{-8}M)	428	448	39	65
Cd (100 μM) + HBR (10^{-8}M)	416	430	28	41
Cd (150 μM) + HBR (10^{-8}M)	304	415	20	39
LSD	18.5	21.3	5.4	6.2

Nodule nitrogen content

The values for nodule nitrogen content increased with the progress of the plant age (Table 4). The treatment with cadmium generated a decline in the values but HBR increased the content of the nitrogen. Moreover, the ill effect generated by 50 μM of cadmium was completely overcome by 10^{-8}M of HBR and the values were comparable with that of the control.

Nodule carbohydrate content

Like that of nodule nitrogen, nodule carbohydrate content (Table 4) exhibited an increase with age of the plants (60 to 90 DAS). Treatment with cadmium decreased the values in a proportion to its concentration (50 to 150 μM). However, HBR (10^{-8}M) improved the carbohydrate content to a significant level and also minimized the damaging effect of the metal.

Carbonic anhydrase activity

As the growth advanced from 60 to 90 days the CA activity increased (Table 5). The activity of the enzyme decreased in the plants fed with cadmium in a ratio with the concentration of the metal. HBR as usual improved the activity to a significant level and also over came the ill effect generated by the metal in a follow up treatment.

Nitrate reductase activity

With the advancement of age (60 to 90 days) the plants exhibited a higher rate of enzyme activity (Table 6) but its level decreased on being fed with cadmium (50, 100, 150 μM). This loss was dependent on the level of the metal (50 to 150 μM). Over all effect of the cadmium was partially

Table 4. Effect of 28-homobrassinolide (HBR) on cadmium (50, 100, 150 μM) induced changes on nodule nitrogen and carbohydrate content in *Cicer arietinum*, at 60 and 90 days, after sowing (DAS).

Treatment	Nodule nitrogen (%)		Nodule carbohydrate (%)	
	60 DAS	90 DAS	60 DAS	90 DAS
Control	3.16	4.31	15.21	18.10
HBR (10^{-8}M)	4.77	5.44	19.13	22.05
Cd (50 μM)	2.96	4.10	15.02	17.44
Cd (100 μM)	2.70	3.81	13.98	15.86
Cd (150 μM)	2.55	3.49	13.10	14.73
Cd (50 μM)+ HBR (10^{-8}M)	3.25	4.51	16.43	19.05
Cd (100 μM) + HBR (10^{-8}M)	2.97	4.19	14.63	16.78
Cd (150 μM) + HBR (10^{-8}M)	2.81	3.71	13.88	16.12
LSD	0.35	0.41	1.15	1.68

Table 5. Effect of 28-homobrassinolide (HBR) on cadmium (50, 100, 150 μM) induced changes in chlorophyll content and carbonic anhydrase [mol $(CO_2)kg^{-1}$ leaf $(F.M)^{-1}S$] activity in the leaves of *Cicer arietinum* at 60 and 90 days, after sowing (DAS).

Treatment	Chlorophyll (mg kg^{-1} F.M)		Carbonic anhydrase [mol $(CO_2)kg^{-1}$(leaf $F.M)^{-1}s$]	
	60 DAS	90 DAS	60 DAS	90 DAS
Control	1.27	2.106	2.93	3.56
HBR (10^{-8}M)	1.94	2.87	3.40	4.25
Cd (50 μM)	1.20	2.05	2.79	3.35
Cd (100 μM)	1.05	1.87	2.58	2.93
Cd (150 μM)	0.93	1.42	2.18	2.27
Cd (50 μM)+ HBR (10^{-8}M)	1.56	2.40	3.12	3.79
Cd (100 μM) + HBR (10^{-8}M)	1.27	2.13	2.61	3.42
Cd (150 μM) + HBR (10^{-8}M)	1.03	1.59	2.14	2.52
LSD	0.14	0.19	0.25	0.29

neutralized by a subsequent treatment with HBR and completely overcame that of 50 μM. The maximum values were records in the plants treated with HBR alone and that were significantly higher from others, including control.

Proline

Compared with the control, the proline content increased in the plants fed with cadmium or HBR (Table 6). Moreover, the plants exposed to the hormone as well as the metal possessed largest quantities which increased further with an increase in the level of the cadmium.

Activity of antioxidative enzymes

The data depicted in table 7 clearly revealed a significant increase in the activity of all the observed antioxidative enzymes (viz – catalase, peroxidase, and superoxide dismutase) in response to growth proceeds from 60 to 90 days and also to the presence of cadmium and/or HBR. Control plants had the minimum values. The activity of the enzymes increased with an increase in the level of metal (50 to 150 μM). Moreover, the spray of HBR to the cadmium treated plants had an additive effect on the enzyme activity.

Yield characteristics and seed protein content

All the yield characteristics, except number of seeds per pod, were significantly affected and exhibited a comparable pattern of response to the treatment (Table 8). The application of the cadmium decreased their values and the loss was in proportion to the increase in the concentration of the metal. However, HBR (10^{-8}M) significantly enriched these characteristics

Table 6. Effect of 28-homobrassinolide (HBR) on cadmium (50, 100, 150 μM) induced changes on nitrate reductase (n mol NO_2 $h^{-1}g^{-1}$F.M) activity and proline content in the leaves of *Cicer arietinum* at 60 and 90 days, after sowing (DAS).

Treatment	Nitrate reductase (n mol $NO_2h^{-1}g^{-1}$F.M)		Proline (mg g^{-1} F.M)	
	60 DAS	90 DAS	60 DAS	90 DAS
Control	298	354	10.68	12.53
HBR (10^{-8}M)	362	470	13.40	17.28
Cd (50 μM)	286	339	12.65	14.55
Cd (100 μM)	263	298	12.78	16.06
Cd (150 μM)	229	275	14.20	18.12
Cd (50 μM)+ HBR (10^{-8}M)	320	382	14.05	18.67
Cd (100 μM) + HBR (10^{-8}M)	291	330	16.10	21.20
Cd (150 μM) + HBR (10^{-8}M)	250	313	16.83	23.05
LSD	29.5	33.4	1.25	1.86

Table 7. Effect of 28-homobrassinolide (HBR) on cadmium (50, 100, 150 μM) induced changes in the activity of catalyase [μ mol H_2O_2 decomposed g^{-1} (F.M)], peroxidase (g^{-1}(F.M.), superoxide (SOD) in *Cicer arietinum* at 60 and 90 days, after sowing (DAS).

Treatment	Catalase (CAT) [μ mol H_2O_2 decomposed g^{-1}(F.M)]		Peroxidase (Pox) [g^{-1}(F.M.)]		Superoxidase (SOD)	
	60 DAS	90 DAS	60 DAS	90 DAS	60 DAS	90 DAS
Control	412	430	14.6	16.5	128	145
HBR (10^{-8}M)	473	509	18.9	22.8	156	190
Cd (50 μM)	440	464	16.3	18.8	138	163
Cd (100 μM)	465	502	17.8	21.3	153	178
Cd (150 μM)	483	547	19.1	23.5	168	209
Cd (50 μM)+ HBR (10^{-8}M)	507	558	21.3	26.1	177	216
Cd (100 μM) + HBR (10^{-8}M)	536	605	26.7	31.4	182	223
Cd (150 μM) + HBR (10^{-8}M)	545	626	27.6	35.0	196	240
LSD	18.5	22.4	1.6	1.9	17.5	16.8

Table 8. Effect of 28-homobrassinolide (HBR) on cadmium (50, 100, 150 μM) induced changes in the number of pods per plants, number of seeds per pod seed yield (g) per plant, 100 seed mass (g) and seed protein content (%) in *Cicer arietinum* at harvest (160 DAS).

Treatment	Number of pods	Number of seeds/pod	Seed yield (g plant^{-1})	100 seed mass (g)	Seed protein content (%)
Control	25.11	2.00	12.38	13.27	19.39
HBR (10^{-8}M)	38.20	2.75	16.28	16.34	21.68
Cd (50 μM)	24.11	2.00	12.05	13.01	19.30
Cd (100 μM)	20.88	1.75	11.96	12.49	18.14
Cd (150 μM)	13.11	1.25	8.81	10.12	17.29
Cd (50 μM)+ HBR (10^{-8}M)	26.55	2.25	13.33	14.38	20.43
Cd (100 μM) + HBR (10^{-8}M)	19.33	2.00	12.29	12.69	18.82
Cd (150 μM) + HBR (10^{-8}M)	15.55	2.00	9.50	11.39	18.03
LSD	3.56	NS	1.56	1.24	0.85

NS = Non-significant

and also overcame the damaging effect of the metal. The hormone given as a followup treatment to the plants already treated with 50 μM of cadmium increased the values to a level equal to that of the control.

The plants treated with cadmium produced the seeds with a lower level of the protein which decreased further with an increase in the concentration of the metal (Table 8). However, the seeds developed on the HBR (10^{-8}M) sprayed plants possessed significantly higher level of the protein. Moreover, per cent protein content in the seeds borned on the plants given cadmium treatment (50 or 100 μM) followed with HBR was statistically equal to that of the control.

Discussion

CHAPTER 5

DISCUSSION

The whole biosphere is currently under an increasing threat of heavy metals that are accumulating in the environment to a level categorized as pollutant. Their presence in the soil is having a direct adverse effect on the growth and development of the plants which is in itself a complex but organized phenomenon. The whole process is determined by various factors borned within or outside the plants. Among them phytohormones occupy an important place, where the brassinosteroids not only act as a natural regulator but also provide protection to the plants against pollutants and the varied stresses (Sasses and Clouse, 1998; Anuradha and Rao, 2001; Rao *et al.,* 2002; Hayat *et al.*, 2003).

The foliage of the plants, exposed to cadmium treatment, exhibited a decline in the activity of carbonic anhydrase (CA) and nitrate reductase (NR) (Tables 5 & 6). This may be an inhibition and/or metabolic dysfunction of the enzyme protein (Hopkins, 1995). Moreover, the metal also has an impact on the activity of plasmamembrane bound proton pump (Obata *et al.*, 1996) and the fluidity of the membrane (Meharg, 1993), restricting the uptake of nitrate, the inducer and the substrate of NR (Harnandez *et al.*, 1996; Compbell, 1999). However, the application of HBR alone or as a followup treatment after feeding cadmium elevated the activity of both CA and NR that could be an expression of the impact of BRs on translation and/or transcription (Khripach *et al.*, 2003). The other possible reason may be the involvement of HBR in elevating the level of NO_3 by

acting at the membrane in case of NR (Mai *et. al.,* 1989) and speeding up of CO_2 assimilation in case of CA (Yu *et al.*, 2004). An increase in the NR and CA activity is in conformity with others (Shen *et al.*, 1990; Singh *et al*, 1993; Hayat *et al.*, 2000a,b, 2001; Hayat and Ahmad, 2003).

The cadmium is known to enhance the level of the enzyme chlorophyllase that brings about the degradation of the chlorophyll (Reddy and Vara, 1986) and also declines the synthesis of 5-aminolauvlinic acid and protochlorophyllide reductase complex (Stobart *et al.*, 1985). Therefore, a cumulative effect that may be generated and expressed in the form of a decrease in the content of chlorophyll (Table 5). This is in conformity with others (Vassileve *et al.*, 1998; Gadallah, 1995). This damaging effect of the metal was overcome in the plants given a followup treatment with HBR (10^{-8} M). The hormone completely neutralized the effect of the lower concentration of cadmium (50 μM) and partially that of higher concentrations (100 and 150 μM). HBR fed to the non-stressed plant, significantly increased the pigment content that is in support of the others (Braun and Wild, 1984; Bhatia and Kaur, 1997; Hayat *et al* 2000, 2001; Fariduddin et al, 2003, and Yu *et al.,* 2004). The reason that sounds best in defending the said observation in possibly the BR-induced impact on transcription and/or translation (Kalinich *et al.,* 1985).

As a natural cause, the plants exposed to stress generate a larger volume of reactive oxygen species (ROS) (Asada, 1999; Dat *et al.*, 2001) that may oxidize protein, lipids and nucleic acids resulting to the abnormalities at the level of the cell (Helliwell and Gutteridge, 1999).

However, the plants are capable to react successfully to such, unnatural conditions by inducing the synthesis of (a) metabolites (ascorbate, glutathione, tocopherole and proline (b) enzymes (superoxide dismutase, catalase, peroxidase, glutathione reductase (Schot *et al.,* 1997; Noctor and Foyer, 1998; Asada, 1999; Schedzendubel and Polle, 2002), that provide additional power of resistance to neutralize the effect of the stress generated activities of peroxidase, catalase, superoxide dismutase and an increase in proline (Table 7). Moreover, the level of these proteins increased further in the plants exposed to cadmium stress in association with a followup treatment with HBR. Similarly, rice plants grown under salinity and supplemented with BRs possessed more protein and the higher activity of antioxidative enzymes (Nunez *et al.*, 2003).

The presence of cadmium in the soil at a stress level is known to decline its microbial population (Rana *et al.*, 2002). Therefore, a decrease in the nodule number, their fresh and dry mass and the activity of the nitrogenase is observed (Table 2 & 3). Thus the metal is said to be most toxic for nitrogen fixation in various group of plants (Kalyanaram and Sivaguranathan, 1993; Kala *et al.*, 1991). However, the phytohormones are recognized to determine establishment, development and also the efficiency of the nodules (Dart, 1977; Hopkins 1995). Among them BRs, being the regulators of transcription and/or translation (Kalinich *et al.,* 1985) and the activity of sucrose synthase (Yu *et al.*, 2004) may have favoured the activity of nitrogenase at the level of the nodule and the formation of nodules (Vardhini and Rao 1999 and Tables 2 & 3). Moreover, BRs also neutralized

the damaging effect of the metal to a limited extent (50 μM). As there is a direct correlation between the activity of nitrogenase and leghemoglobin content (Dakora, 1995; Comba *et al.*, 1997; 1998), therefore the contents of leghemoglobin also decreased as the concentration of the cadmium fed to the plants was increased from 50 μM to 150 μM (Table 3). However, HBR alone or as a followup treatment improved the level of leghemoglobin and that may be because of the same reasons as mentioned earlier for nitrogenase activity. A decline in the level of the nodule nitrogen and carbohydrate (Table 4) induced by the presence of the cadmium is possibly the adverse effect of the metal on the cellular metabolism (Prasad, 1995) and the permeability of the membrane (Prasad, 1995). However, the regulatory role of HBR has possibly normalized the above factors to make available more and more nitrogen and carbohydrates for speeding up the activity of the bacteria.

In the earlier writing it has been mentioned that the damage caused by the presence of cadmium is of a significant level that could have been expressed further at the level of the shoot growth (Table 1). The fresh and dry mass of the plants decreased with an increase in the level of the metal (50 to 150 μM). This supports the earlier observations in soybean (Cataldo *et al.,* 1983; Dowdey and Ham, 1997). An additional reason that may simplify the cause behind this damage may be the loss of cellular turgor, inhibiting the activity of the cell and its enlargement (Gabbrielli *et al.*, 1990). Like that of other parameters, HBR also improved the fresh and dry mass of the plants (Table 1) because they also proved to be essential for

growth in the mutent of *Arabidospis thaliana* (Li *et al.*, 1996) and *Pisum sativum* (Nomuro *et al.*, 1997). Therefore, the exogenous application of BRs to varied group of plants is found to improve the growth of the root and/or that of shoot (Gregory 1981, Mandava and Sasse, 1981; Katsumi, 1985; Mandava, 1988; Dahse *et al.,* 1991; Clouse *et al.*, 1991; Schilling *et al.,* 1991; Petzold, 1992; Singh *et al.*, 1993; Sasse, 1996; Bajguz *et al.*, 1996; Vardhini and Rao, 1998; Fujioka, 1999; Hayat *et al.*, 2001; Bajguz, 2003; Fariduddin *et al.,* 2004, 2005,).

A decrease in the seed yield per plant, the weight of 100 seeds and seed protein content in the plants, treated with cadmium is possibly the fall out of the poor growth and nodulation (Tables 1 & 2). It may, however, be overcome completely, at least in those plants fed with 50 μM of the metal, by the follow up treatment with HBR because not only that the hormone has improved various characteristics (mentioned above) but also favours mineral and water uptake (Sairam, 1994) assimilation of nitrate (Mai *et al.*, 1989), photosynthesis (Mai *et al.*, 1989, Shen *et al.*, 1990; Hayat *et al.*, 2001a) and protein synthesis (Kalinich *et al.*, 1985; Mandava, 1988; Bajguz, 2000).

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Appendix

APPENDIX

1 Preparation of reagents for leghemoglobin estimation

1.1 Sodium phosphate buffer (pH 7.4) :

It was prepared by separately dissolving 13.9 g of NaH_2PO_4 and 26.82 g of Na_2HPO_4 in sufficient DDW to make the volume of each solution to 1000 cm^3. These solutions were mixed in the ratio of 19:81, respectively.

1.2 Alkaline pyridine reagents :

It was prepared by dissolving 0.8 g of NaOH in 50 cm^3 of DDW and allowed to cool. 33.8 cm^3 of pyridine was added to it and diluted to 100 cm^3 with DDW. This produced 4.2M pyridine in 0.2M NaOH.

2 Reagents for nitrogen estimation

2.1 2.5N NaOH

5g of NaOH was dissolved in sufficient DDW and final volume was maintained upto 100 cm^3.

2.2 Sodium silicate (10%)

10g sodium silicate was dissolved in 100 cm^3 DDW.

3 Preparation of reagents for carbohydrate estimation

3.1 1.5 N H_2SO_4

40.8 cm^3 of concentrated H_2SO_4 (AR) was pipetted into sufficient DDW and final volume was made upto 1000 cm^3, using DDW.

3.2 Phenol (5%)

50g of distilled phenol was dissolved in sufficient DDW and final volume was made upto 1000 cm^3 by DDW.

4 Preparation of reagents for nitrate reductase activity

4.1 0.1M phosphate buffer (7.4 pH)

27.2 g of KH_2PO_4 and 45.63 g of $K_2HPO_4.7H_2O$ were dissolved separately in 1000 cm^3 of DDW. The above solutions of KH_2PO_4 and $K_2HPO_4.7H_2O$ were mixed in the ratio of 16:84, respectively.

4.2 0.2M potassium nitrate

20.2 g of KNO_3 was dissolved in sufficient DDW and final volume was made upto 1000 cm^3, using DDW.

4.3 Isopropanol (5%)

5 cm^3 of isopropanol was pipetted into sufficient DDW and final volume was made upto 100 cm^3, using DDW.

4.4 Sulphanilamide (1%)

1g of sulphanilamide was dissolved in 100 cm^3 of 3N HCl. 3N HCl was prepared by dissolving 25.86 cm^3 of HCl insufficient DDW and final volume was maintained to 100 cm^3, by using DDW.

4.5 N-1-nephthyl-ethylenediamine dihydrochloride (NED-HCl) (0.02%)

20 mg of NED-HCl was dissolved in sufficient DDW and final volume was made upto 100 cm^3, by using DDW.

5 Preparation of reagents for the estimation of carbonic anhydrase activity

5.1 Cystein hydrochloride solution (0.2M)

4.8 g of cystein-HCl was dissolved in sufficient DDW and final volume was made upto 1000 cm^3, by using DDW.

5.2 Sodium phosphate buffer

27.8 g NaH_2PO_4 and 53.65 Na_2HPO_4 was dissolved each separately in sufficient DDW and final volumed was made 1000 cm^3. 51 cm^3 of NaH_2PO_4 and 49 cm^3 of Na_2HPO_4 were then mixed to get the required solution.

5.3 Alkaline sodium bicarbonate solution

16.8 g sodium bicarbonate ($NaHCO_3$) was dissolved in aqueous 0.2M NaOH solution [0.8 g NaOH (1000 cm^3)$^{-1}$] and final volume was made upto 100 cm^3, by using DDW.

5.4 Bromothymol blue (0.002%)

0.002g of bromothymol blue was dissolved in sufficient DDW and final volume was made upto 100 cm^3, by using DDW.

5.5 0.01N HCl

0.86 cm^3 of pure HCl was pipetted in sufficient DDW and final volume was made upto 1000 cm^3 by using DDW.

5.6 Methyl-red indicator

A pinch of methyl red was dissolved in sufficient ethanol and final volume was made 100 cm^3 by using ethanol.

6 Reagent for chlorophyll estimation

80% acetone was prepared by mixing 80 cm^3 of acetone with 20 cm^3 of DDW.

7 Preparation of reagents for proline estimation

7.1 Sulphasalicylic acid (3%)

3g of sulphasalicylic acid was dissolved in sufficient DDW and final volume was maintained 100 cm^3, by using DDW.

7.2 Acid ninhydrin solution

1.25 g of ninhydrin was dissolved in a mixture of warm, 30 cm^3 of glacial acetic acid and 6M phosphoric acid (pH 1.0) with agitation till it got dissolved. It was stored at 4°C and used within 24 h.

The 6M phosphoric acid was prepared by mixing 11.8 cm^3 of phosphoric acid with 8.2 cm^3 of DDW.

8 Reagent for peroxidase estimation

8.1 Pyrogallol phosphate buffer

It was prepared by mixing 25 ml of pyrogallol in 75 ml phosphate buffer (pH 6).

9 Reagents for catalase estimation

9.1 Phospahte buffer (0.1M) for pH 6.8

3.54 g of Na_2HPO_4 was dissolved in 100 cm^3 of DDW and 3.72 g of NaH_2PO_4 was added to 100 cm^3 of DDW. To this 12.3 cm^3 of Na_2HPO_4 was added to 87.7 cm^3 of NaH_2PO_4.

9.2 H_2O_2 (0.1 M)

0.34 cm^3 of H_2O_2 was added to 100 cm^3 of distilled water.

9.3 Sulphuric acid H_2SO_4 (2%)

2 cm^3 of H_2SO_4 was added to 98 cm^3 of DDW.

9.4 0.1N potassium permanganate

This was made by dissolving 0.162 g of $KMnO_4$ in 500 cm^3 of distilled water.

10 Reagents for superoxide dismutase

10.1 Phosphate buffer (50 mM) for pH 7.8

It was prepared by mixing 1.78 g Na_2HPO_4 and 1.56 g of NaH_2PO_4 in 100 ml of DDW separately and mixing 91.5 ml of Na_2HPO_4 with 8.5 ml of NaH_2PO_4.

10.2 Methionine (13 mM)

It was prepared by dissolving 0.193 g of methionine in 100 ml of DDW.

10.3 Nitrobluetetrazelium (NBT) (75 μM)

6.13 mg of NBT was dissolved in 100 ml of DDW.

10.4 Riboflavin (2M)

0.732 mg of riboflavin was dissolved in 100 ml of DDW.

10.5 EDTA (0.1M)

2.92 g EDTA was dissolved in 100 ml of DDW.

11 Preparation of reagents for protein estimation

11.1 Trichloroacetic acid (TCA) (5%)

5 cm^3 of TCA was mixed with 95 cm^3 of DDW.

11.2 1N NaOH

40 g of NaOH was dissolved in sufficient DDW and final volume was made upto 1000 cm^3, by using DDW.

11.3 Reagent A : 2% sodium carbonate (2g dissolved in 100 cm^3 DDW) and 0.1N NaOH (4 g NaOH dissolved in 1000 cm^3) were mixed in the ratio 1:1.

Reagent B : 0.5% copper sulphate (500 mg $CuSO_4$ dissolved in 100 cm^3) and 1% sodium tartarate (1 g sodium tatrate dissolved in 100 cm^3 DDW) were mixed in the ratio 1:1.

Reagent C : 50 cm^3 of reagent A was mixed with 1 cm^3 of reagent B, except omission of sodium hydroxide.

11.4 Folin phenol reagent (1N of an acid)

The reagent obtained from Loba Chemie Pvt. Ltd. Mumbai, India was diluted with DDW in the ratio 1:2.